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A mild and straightforward one-pot hyaluronic acid functionalization through termination of poly-(2-alkyl-2-oxazoline)

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ABSTRACT

Hyaluronic acid (HA) of different molar masses from 38 000 to 990 000 g.mol⁻¹ were used as terminating agents during the cationic ring-opening polymerisation of 2-methyl-2-oxazoline. HA was first converted into a tetrabutylammonium salt to solubilize it in polar aprotic organic solvents where the termination was conducted by varying the excess of HA units to the growing poly(2-methyl-2-oxazoline) (PMeOx) chains (with various PMeOx degree of polymerisation). This led to HA derivatives with various PMeOx degrees of substitution from 4 % up to 13 % (determined by ¹H NMR). HA-g-PMeOx copolymers were analysed by Size Exclusion Chromatography with on-line detectors (Multi-angle light scattering, Viscometer, Refractive index) in 0.1 mol.L⁻¹ LiNO₃ to study their macromolecular characteristics in dilute medium. The reaction was applied to functionalize HA with thermo-responsive poly(2-isopropyl-2-oxazoline)-co-poly(2-*n*-butyl-2-oxazoline) moieties, resulting in materials displaying thermally dependent hydrogel properties in aqueous solutions which could be used for biomedical applications.

Keywords: Hyaluronic acid; Poly(2-alkyl-2-oxazoline); Grafting to; One-pot reaction; Hydrogels

1. Introduction

Among polysaccharides, hyaluronic acid (HA) is a material of choice for biomedical applications as it is a component of the extracellular matrix and synovial fluid in vertebrates (including humans) and some bacteria, hence its perfect biocompatibility. HA is the only non-sulfated member of the glycosaminoglycan family [1][2]. It has a regular (1→4)-linked disaccharide unit which consists of alternating β -D-glucuronic acid (1→3)-linked to *N*-acetyl- β -D-glucosamine. It is involved in several physiological functions such as cell motility, proliferation mechanisms, inflammatory response and articulation lubrication. In particular, HA is used for treating osteoarthritis and as a wound healing promoter [1]. Its specific interaction with cell receptor CD44, which is overexpressed in some cancerous cells, has also led to many research articles probing its use in drug delivery systems [3]. In comparison to many other polysaccharides, HA allows a wider range of chemical modifications thanks to both its alcohol [3] and carboxylic acid groups [1] [2]. Carboxylic acid moieties also strongly influence the solubility of HA depending on the counter ion accompanying the carboxylate anion. Sodium hyaluronate (Na-HA, the sodium salt form of HA) is water-soluble and insoluble in any organic solvents contrary to tetrabutylammonium hyaluronate (TBA-HA, the tetrabutylammonium salt form of HA) which is soluble in polar aprotic solvents such as DMSO and DMF [4].

An ongoing challenge is to functionalize HA with suitable grafting moieties or with suitable polymers to combine both the properties of HA and of grafted groups. “Grafting from” and “grafting to” are the two main pathways to connect polymer chains on a backbone. In a “grafting from” approach, the polysaccharide is used as a macro-initiator for polymerisation [5] with the advantage that steric hindrance effect will be minimized compared to a “grafting to” approach, resulting in a generally higher reaction efficiency [6]. The drawback is that the resulting growth of the polymer chain might not be as well-controlled as with a “grafting to” reaction scheme because of the use of a macro-initiator, which can be problematic when the structure is closely related to the polymer properties [7]. Also, the characterisation of the lateral chains will be more difficult. In the “grafting to” approach, the grown polymer chain is directly grafted to the polysaccharide [3][8], allowing a better control of the resulting copolymer structure. In this study, the “grafting to” approach was selected to finely design the final material properties according to the grafted polymer structure. Nevertheless, this functionalization pathway requires at least 2 steps (i.e. polymerisation of a monomer and its subsequent grafting to the polysaccharide), sometimes even a supplementary step if an end-

chain functionalization is needed. Therefore, new approaches leading to a reduction of the synthesis steps are of great interest.

Among grafted polymers, poly(ethylene glycol) (PEG) is one of the most used due to its stealth properties making it untargeted by the immune system which is useful for designing drug delivery materials. However, PEG is prone to oxidation [9] and to accumulate in organs [10]. Furthermore, its manufacturing requires the use of highly toxic and hazardous ethylene oxide monomer [9]. Polylactide-grafted polysaccharides copolymers are also good candidates for biomedical or pharmaceutical applications because polylactide is biodegradable and can be obtained from renewable sources [11][12]. For example, polylactide-grafted dextran has been synthesized by the “grafting from” pathway in a three steps reaction involving a partial reversible protection of the polysaccharide hydroxyl functions, the ring opening polymerization (ROP) of D,L-lactide catalysed by stannous octoate and promoted by the remaining OH groups of the partially protected dextran and finally a deprotection step [11]. Due to its toxicity, the presence of stannous salts remains a major problem for biomedical applications. Poly(2-alkyl-2-oxazoline)s (POx) constitute alternative polymers for biomedical applications due to their biocompatibility and their stealth properties [13]. They display other interesting features such as modular physical properties (value of glass temperature, amorphous or semi-crystalline nature, solubility) as well as thermo-responsiveness [7] depending on the nature of the pendant alkyl group. The grafting of POx on polysaccharides have already been undertaken using a “grafting to” approach by Aoi *et al.* on chitosan. The amino groups of chitosan acted as terminating agents for the growing POx chains in DMSO [14] [15]. They focused first on homopolymers: poly(2-methyl-2-oxazoline) and poly(2-ethyl-2-oxazoline) [14] before extending their reaction to an amphiphilic poly(2-phenyl-2-oxazoline)-*b*-poly(2-methyl-2-oxazoline) block copolymer [15]. However, this synthetic approach cannot be extended to HA, which is lacking amine functionality. In another approach, Hruby and coll. focused on the functionalization of glycogen and β -glucan with thermo-responsive poly(2-isopropyl-2-oxazoline)-*co*-poly(2-*n*-butyl-2-oxazoline) [16] [17] [18]. The polysaccharides were first activated by converting their alcohol moieties into alcoholate using a strong base, sodium hydride, to be nucleophilic enough to induce the termination step. However, the drawback of this approach is the requirement to use sodium hydride in DMSO which is preventing the reaction scale-up, the combination of both being potentially hazardous on larger quantities [19]. Also, extending this reaction to HA might be challenging, as this polysaccharide has been

proven to be quite easily degraded in the presence of bases [20], which questions its compatibility with sodium hydride. As an alternative route for grafting of POx on HA without using sodium hydride, we thought we could employ the carboxylic acid groups of HA as terminating agents for the cationic ring-opening polymerisation (CROP) of oxazolines as these functional groups have previously been used for such purpose with either small organic molecules like methacrylates [21] [22] or synthetic polymers like poly(methyl methacrylate)s [23].

In summary, our study focus on the use of HA as a terminating agent during the CROP of 2-methyl-2-oxazoline (MeOx) to obtain HA grafted with poly-(2-methyl-2-oxazoline) (PMeOx) named HA-*g*-PMeOx copolymers. We varied the HA/PMeOx ratios and the grafted PMeOx chain length (with the DP_{PMeOx}) and checked their grafting amounts and the molar masses of HA. HA-*g*-PMeOx copolymers have been characterised by FTIR and NMR spectroscopies. PMeOx and HA being very hydrophilic, the successful grafting has been readily assessed using both Size Exclusion Chromatography (SEC) with on-line Multi-Angle Light Scattering system (MALS), Viscosity Detector (VD) and Differential Refractive Index detector (DRI) in aqueous media and Diffusion-Ordered Spectroscopy (DOSY) NMR. The reaction was then extended to the termination of HA with well-known thermo-responsive poly(2-isopropyl-2-oxazoline)-copoly(2-*n*-butyl-2-oxazoline) (PiPrOx-co-P*n*BuOx) [7] [16] in order to design physical hydrogels whose formation would be triggered by temperature increase.

2. Experimental

2.1. Materials

Three different Na-HA batches were kindly provided by Givaudan France SAS (Pomacle, France): CRISTALHYAL® $M_n = 990\,000\text{ g.mol}^{-1}$ (HA_{990k}), BASHYAL® $M_n = 140\,000\text{ g.mol}^{-1}$ (HA_{140k}) and RENOVHYAL® $M_n = 38\,000\text{ g.mol}^{-1}$ (HA_{38k}) (absolute molar masses were determined through SEC/MALS/VD/DRI measurement). 2-methyl-2-oxazoline, 2-*n*-isopropyl-2-oxazoline (*i*PrOx), 2-*n*-butyl-2-oxazoline (*n*BuOx), methyl *p*-toluenesulfonate, anhydrous DMSO, anhydrous acetonitrile, sodium chloride, tetrabutylammonium hydroxide (54.0-56.0 % in water) (TBAOH), phosphate buffer solution (PBS) tablets, deuterium oxide (D₂O), sodium deuterioxide solution (30 wt% in deuterium oxide), deuterated chloroform (CDCl₃) were purchased from Sigma-Aldrich. Hydrochloric acid 36.5 %, sodium hydroxide pellets, amberlite

ion exchange resin (IRN77, cationic type, H⁺), acetone and ethanol 96% vol% were purchased from VWR. Water was purified with the milli-Q water reagent system (Millipore, USA).

2.2. Conversion of Na-HA to TBA-HA

The principle of the conversion of Na-HA to TBA-HA is summed up in scheme 1 (a). The three different HAs (HA_{990k}, HA_{140k} and HA_{38k}) were first transformed into their acidic form: 5 g of Na-HA were first dissolved overnight in 830 mL of water at room temperature. Then the cation exchange resin (H⁺) (45 g) was introduced in the solution, which was left to stir for one day at room temperature. The resin was subsequently filtered off the solution through a sintered glass filter. The resulting solution was then neutralized using TBAOH to obtain TBA-HA as follow: TBAOH, diluted fivefold with water was added drop-wise to the previously prepared hyaluronic acid solution until the pH reached 8. The solution was then freeze-dried and the resulting TBA-HA was kept in a freezer until further use.

2.3. One-pot functionalization of TBA-HA with PMeOx through a “grafting to” approach

2.3.1. Polymerisation of MeOx (DP: 30 and 50)

The CROP protocol is presented in scheme 1 (b). MeOx (2.056 g, 24.16 mmol) was introduced in an oven-dried round-bottom flask under nitrogen containing a solution of methyl-*p*-toluenesulfonate (DP 30: 150 mg, 0.805 mmol; DP 50: 90 mg, 0.483 mmol) in 6 mL dry acetonitrile. Nitrogen was bubbled within the mixture for 15 minutes at room temperature after which the polymerisation mixture was stirred at 70 °C under nitrogen overnight. DPs were checked by ¹H NMR (see Fig. S1) and calculated according to Eq. (S1). For the polymerisation of PiPrOx-co-PnBuOx copolymers, the same protocol (target DP: 30) was applied by substituting MeOx with both *i*PrOx (2.19 g, 19.4 mmol) and *n*BuOx (615 mg, 4.8 mmol) which were added simultaneously to the flask containing the initiator (target *i*PrOx/*n*BuOx ratio: 80/20; experimental ratio: 75/25 as determined with NMR).

2.3.2. Termination of PMeOx with TBA-HA

The termination step is presented in scheme 1 (c) and described herein for PMeOx DP 30. For PMeOx DP 50, the terminating agent TBA-HA molar quantity was adjusted to the PMeOx polymer chain molar amount which was assumed to be equal to the initiator molar amount introduced during the PMeOx polymerisation step (see section 2.3.1.).

mixture to exchange the TBA counter-ions with sodium. The modified polysaccharide was subsequently precipitated in acetone, then filtered and immediately placed in a 0.2 mol.L⁻¹ NaCl solution (100 mL per gram of starting TBA-HA) to substitute the remaining TBA counter-ions with sodium. After one night, the resulting solution was dialyzed against distilled water (dialysis advance was monitored with conductivity measurement, dialysis membrane cut off (Repligen, MA, USA) was adjusted to starting polysaccharide: 6-8 kDa for TBA-HA_{38k} and 12-14 kDa for the two other batches). The material was recovered through freeze-drying. It was subsequently washed by dispersing it in ethanol and recovering it through filtration before drying it under vacuum, the process being performed three times to ensure removal of remaining free PMeOx chains. Degrees of substitution (DS) were determined by ¹H NMR (see section 2.4.2.). For the termination of HA with PiPrOx-co-PnBuOx, the same protocol was applied with a PiPrOx-co-PnBuOx (polymer chain): TBA-HA (disaccharide unit) molar ratio of 1:1.

Finally, the samples will be coded as follow: HA_{M_n}-g-PMeOx_{DP}-DS (HA_{M_n}-g-(PiPrOx_{DP(PiPrOx)}-co-PnBuOx_{DP(PnBuOx)})-DS for the thermo-responsive material), where M_n is the number average molar mass of HA (e.g. 140k for 140 000 g.mol⁻¹), DP the degree of polymerisation of PMeOx (DP(PiPrOx) and DP(PnBuOx) the DPs of PiPrOx and PnBuOx within the copolymer) and DS the degree of substitution in grafted PMeOx.

2.4. Characterisation methods

2.4.1. Infrared spectroscopy

Infrared spectra were acquired on a spectrometer Nicolet IS50 FT-IR (Thermo Scientific, USA). The samples were analysed with the OMNIC software by transmission from 500 to 4000 cm⁻¹ (32 scans with resolution 2 cm⁻¹).

2.4.2. NMR spectroscopy

¹H NMR spectra were acquired on a Bruker Avance 300 MHz spectrometer (MA, USA). The PMeOx homopolymer samples were prepared in CDCl₃ while HA derivatives were prepared at a concentration of 5 g.L⁻¹ in D₂O containing 0.125 M sodium deuterioxide NaOD to increase HA protons mobility, thus improving spectra resolution [24].

The experimental grafting degree of PMeOx to HA (DS) was estimated by ¹H NMR spectroscopy using Eq. (1).

$$DS = \frac{2 \times (I_{Methyl} - 3)}{3 \times DP_{PMeOx} \times I_{Hanom}} \quad (1)$$

Where I_{Methyl} is the integration value of the overlapping peaks associated to methyl from HA and PMeOx respective *N-acetyl* moieties found at 1.8-2.1 ppm. DP_{PMeOx} stands for the experimental degree of polymerisation of MeOx (determined by 1H NMR spectroscopy before the termination step according to Eq. (S1)). I_{Hanom} accounts for the two anomeric protons of the disaccharide unit found near 4.5 ppm which were used as a reference peak as they were not overlapping with PMeOx or other HA proton peaks (Fig. 1).

DOSY NMR measurements were performed on a Bruker Avance III 600 MHz equipped with a CPTCI z-gradient probe (maximum gradient power of 50 G.cm⁻¹). The details on the experiment will be covered in the supplementary information.

2.4.3. SEC/MALS/VD/DRI

The SEC/MALS/VD/DRI measurements were conducted as follow. The carrier was a 0.1 mol.L⁻¹ LiNO₃ solution filtered through a 0.1 µm filter unit (Millipore, USA), degassed on-line (DGU-20A3, Shimadzu, Japan), and eluted at a 0.5 mL.min⁻¹ flow-rate (LC10Ai Shimadzu, Japan). The analysed solutions were prepared in 0.1 mol.L⁻¹ LiNO₃ at a concentration of respectively 0.5 g.L⁻¹ (HA_{990k} based materials) and 1 g.L⁻¹ (HA_{140k} and HA_{38k} based materials) and were filtered on a 0.45 µm filter prior to injection of 100 µL with an automatic injector (SIL-20A Shimadzu, Japan). The SEC line consisted of an OHPAK SB-G guard column for protection and two OHPAK SB 806 and 804 HQ columns (Shodex Showa Denko K.K., Japan) in series. The column packing was a poly(2-hydroxyethyl methacrylate) gel. The MALS detector was a DAWN Heleos-II (Wyatt Technology Inc., CA, USA) fitted with a 50 µL K5 cell and 18 photodiodes (normalized relative to the 90° detector using bovine serum albumin). The viscometer detector (VD) was a ViscoStar II (Wyatt Technology Inc., CA, USA). The collected data were analysed using the Astra® 6.1.7.16 software package. Molar masses were obtained with the Zimm order 1 method. The concentration of each eluted fraction was determined with DRI (RID 10A Shimadzu, Japan) according to the values of dn/dc (0.150 mL.g⁻¹ for HA and 0.158 mL.g⁻¹ for PMeOx), which were determined through DRI measurements using polymer solutions in 0.1 M LiNO₃ at different concentrations. The dn/dc of the HA-*g*-PMeOx samples were then calculated as the average of the dn/dc of HA and PMeOx according to the weight content of the respective polymers in the copolymer.

2.4.4. Rheology

Rheological measurements were performed on a Discovery HR-2 hybrid rheometer from TA Instruments (UK). HA_{990k}-g-(PiPrOx₂₀-co-PnBuOx₇)-0.10 was dissolved in PBS (0.01 M phosphate buffer, 0.15 M NaCl, pH: 7.4) at a concentration of 15 g.L⁻¹. The measurements were performed with a double concentric cylinder measuring system and the rheometer was operated using the TRIOS v4 software.

3. Results and discussion

3.1. Conversion of Na-HA to TBA-HA

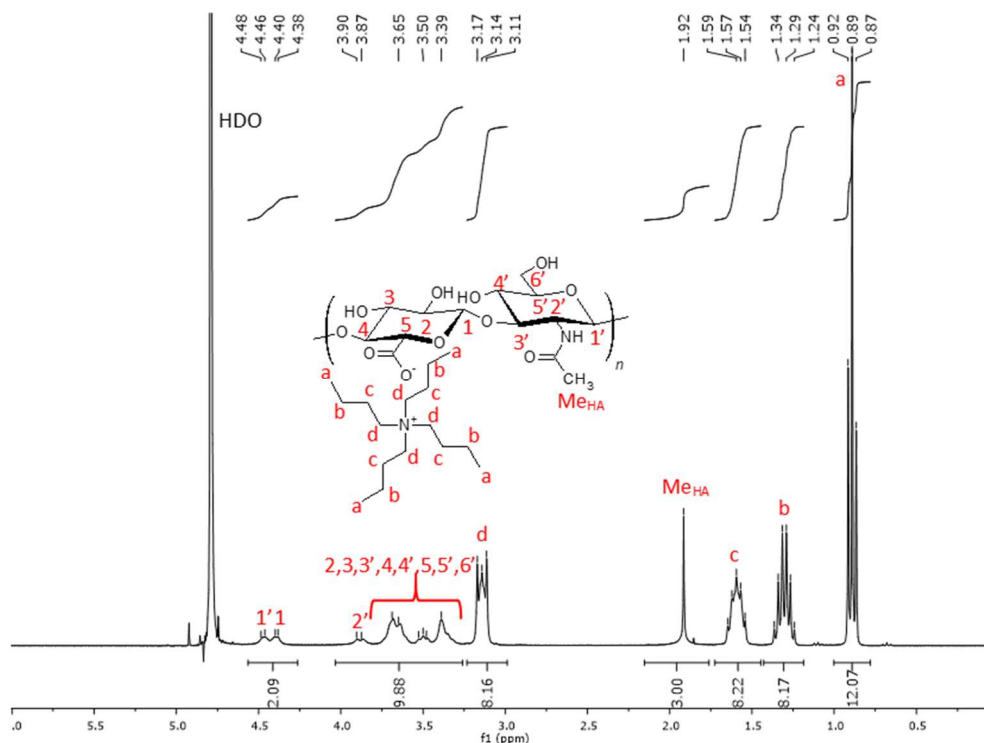
TBA-HA was obtained through a two-steps process (scheme 1a). Na-HA was first acidified with an amberlite ion-exchange resin (cationic type) and then neutralized with TBA-OH. The resulting TBA-HA was analysed by ¹H NMR spectroscopy to assess the full conversion of Na-HA to TBA-HA (Fig. 1). Me_{HA} (δ=1.92 ppm), corresponding to the three protons of the methyl group of HA, was taken as the reference peak. A complete conversion into TBA-HA was calculated from either the H_b, H_c or H_d protons of TBA at respectively 3.14, 1.58 or 1.29 ppm (integration value of 8 indicating full conversion) or the H_a protons of TBA at 0.89 ppm (integration value of 12 indicating full conversion).

SEC/MALS/VD/DRI analysis were conducted on Na-HA and TBA-HA salts in LiNO₃ 0.1 mol.L⁻¹ as summarised in Table 1. It clearly appeared that both Na-HA and subsequent TBA-HA salts presented quite the same molar masses. For instance, Na-HA_{140k} had a M_n of 140 000 g.mol⁻¹ and a M_w of 210 000 g.mol⁻¹ which respectively shifted to 160 000 g.mol⁻¹ and 250 000 g.mol⁻¹ when converted into TBA-HA, which was within the margin of error of the measurement.

Table 1. Evolution of HA molar masses induced by the salt substitution reaction using SEC/MALS/VD/DRI.

Sample	M _n (g.mol ⁻¹) (Na-HA)	M _w (g.mol ⁻¹) (Na-HA)	M _n (g.mol ⁻¹) (TBA-HA)	M _w (g.mol ⁻¹) (TBA-HA)
HA _{38k}	38 000	64 000	36 000	68 000
HA _{140k}	140 000	210 000	160 000	250 000
HA _{990k}	990 000	1 500 000	1 000 000	1 370 000

255 Accuracy of SEC/MALS/VD/DRI data: $M_n \pm 9\%$; $M_w \pm 6\%$.



257 **Fig. 1.** ^1H NMR spectrum of TBA-HA_{140k} in D₂O (with 0.125 M NaOD).

259 3.2. PMeOx grafting to HA: investigation of a one-step process

260 PMeOx was polymerised with an experimental DP of either 29 (Fig. S1) or 47 and
 261 subsequently terminated with TBA-HA_{38k}, TBA-HA_{140k} or TBA-HA_{990k} by varying the PMeOx chain
 262 to HA unit molar ratio (1:2 and 1:1) as summarized in Table 2. The nucleophilic termination of
 263 CROP grown PMeOx was expected to be induced by the coupling of the cationic oxazolinium
 264 end-group from the active propagating species on the anionic carboxylate moieties of HA, as
 265 carboxylates have already been used as terminating agents during oxazoline polymerisation
 266 [21]. Also, alcoholic moieties were not activated by turning them into alkoxide and were
 267 therefore assumed not to be nucleophilic enough for terminating propagating oxazolinium.
 268 The reaction was thus assumed to follow a similar pattern as the one described during
 269 esterification of HA or alginate using alkyl halides (Scheme 1c) [4] [25]. The reaction was
 270 stopped by adding an excess of sodium chloride solution to convert TBA-HA-*g*-PMeOx into Na-
 271 HA-*g*-PMeOx, with the material being precipitated afterwards in acetone, recovered through
 272 filtration and then purified by dialysis and several washings with ethanol.

FTIR analysis of resulting HA-*g*-PMeOx revealed characteristic bands of both starting HA at 3610-2970 cm^{-1} (O-H stretching), 2888 cm^{-1} (C-H stretching), 1606 cm^{-1} (carboxylate C=O asymmetric stretching), 1558 cm^{-1} (N-H bending), 1404 cm^{-1} (carboxylate C=O symmetric stretching), 1318 cm^{-1} (carboxylate C-O stretching) and 1033 cm^{-1} (C-OH stretching), and of PMeOx at 1620 cm^{-1} (tertiary amide C=O stretching), 1420 cm^{-1} (amide C-N stretching), 1240 cm^{-1} (backbone C-N stretching) [25] with an additional signal at 1739 cm^{-1} which corresponds to the ester bond between HA and PMeOx (Fig. 2). Qualitatively, the grafting appears successful.

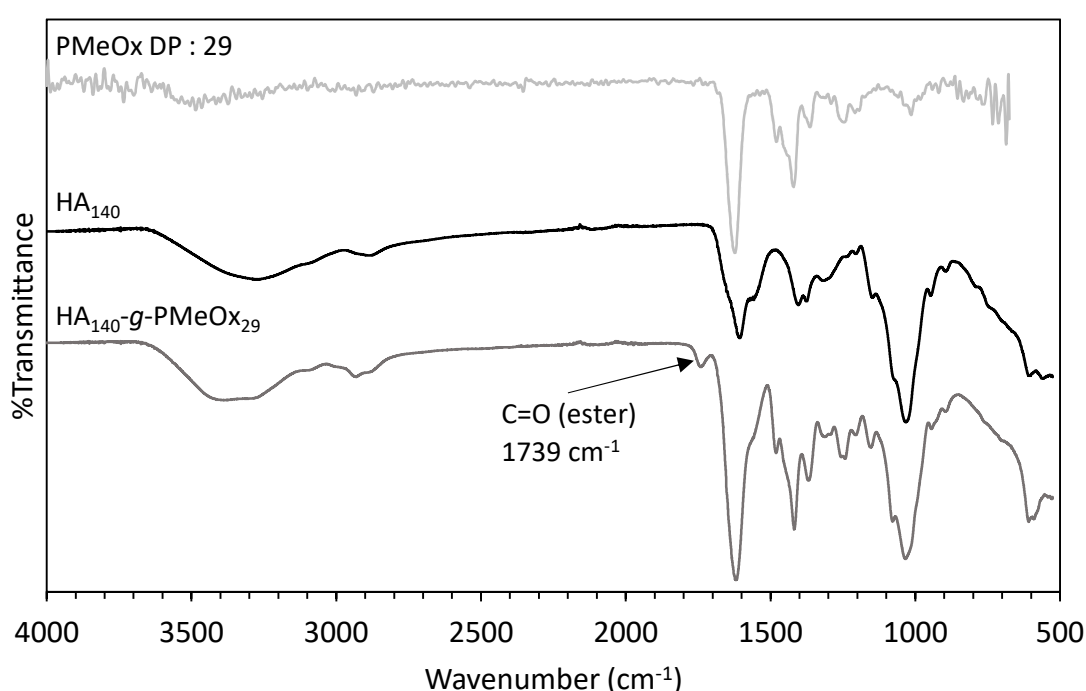


Fig. 2. FTIR spectra of PMeOx DP: 29 terminated with KOH (1 M in MeOH) (top, light grey) HA_{140k} (middle, black) and HA_{140k}-*g*-PMeOx₂₉-0.13 (bottom, dark grey).

The NMR spectrum (Fig. 3) also displayed peaks of HA (anomeric protons (1 and 1') at 4.40-4.50 ppm, proton in α position of amide (2') around 3.9 ppm, the other protons from the sugar ring between 3.44 and 3.71 ppm, methyl Me_{HA} at 1.94 ppm), as well as peaks of PMeOx (methylene protons (b) at 3.44 ppm, methyl end group (Me_{endgr}) at 3.04-3.08 ppm and methyl groups (Me_{PMeOx}) around 2.1 ppm). The grafting degree (DS) was estimated according to Eq. (1), the results being summed up in Table 2. The highest obtained DS was 13% (for sample HA_{140k}-*g*-PMeOx₂₉-0.13 displayed in Fig. 3), which means 45 % of the weight of this material actually corresponds to PMeOx as calculated with Eq. (2).

$$Wt\%_{PMeOx} = \frac{M_{n_{PMeOx}} \times DS}{DS \times (M_{n_{PMeOx}} + M_{u_{HA}} - M_H) + (1 - DS) \times M_{u_{NaHA}}} \times 100 \quad (2)$$

Where $M_{n_{PMeOx}}$ is the number average molar mass of PMeOx chain calculated by 1H NMR spectroscopy using the following formula: $M_{n_{PMeOx}} = DP_{PMeOx} \times M_{MeOx} + M_{endgr}$ where $M_{MeOx} = 85 \text{ g.mol}^{-1}$ and $M_{endgr} = 15 \text{ g.mol}^{-1}$ (see Eq. (S1) and Fig. S1). DS is the grafting degree of PMeOx on HA, $M_{u_{HA}}$ is the molar mass of the acid form of HA disaccharide unit, M_H the molar mass of hydrogen and $M_{u_{NaHA}}$ the molar mass of the sodium form of HA disaccharide unit.

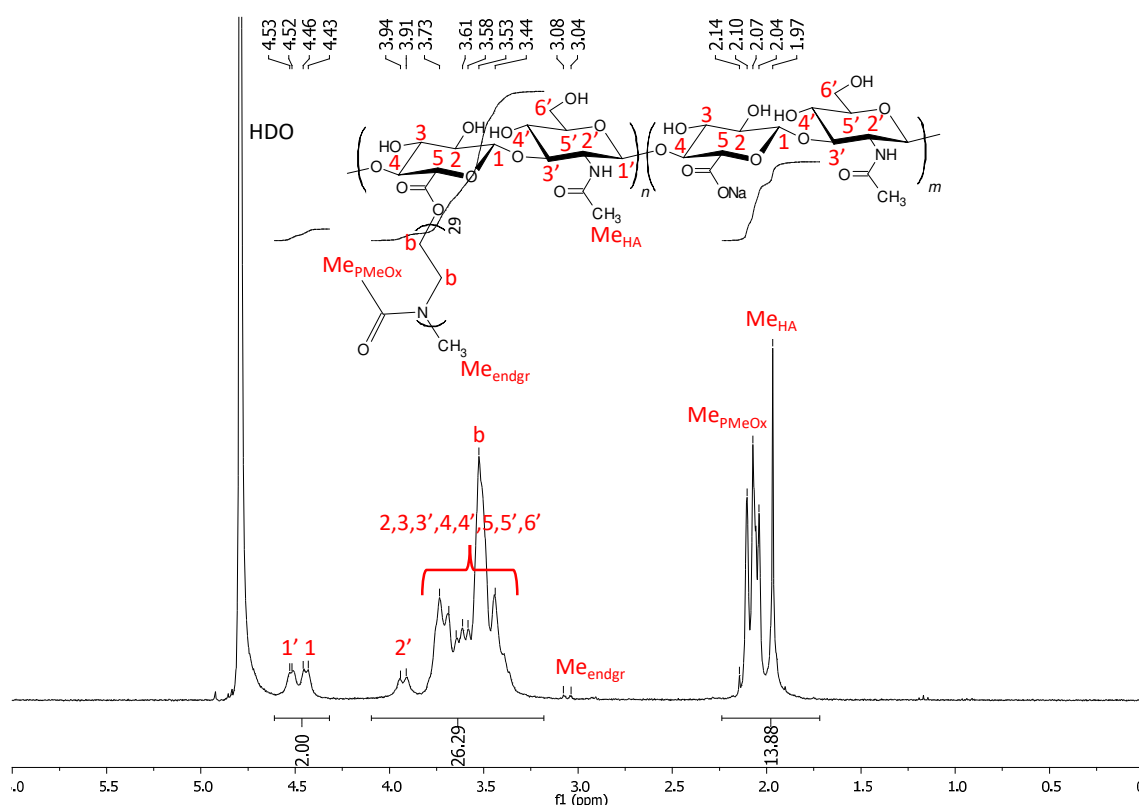


Fig. 3. 1H NMR spectrum of HA_{140k}-g-PMeOx₂₉-13% in D₂O (with 0.125 M NaOD).

The grafting efficiencies were ranging from 8 to 16 %, except with sample HA_{990k}-g-PMeOx₂₉-0.12 having a surprisingly higher grafting efficiency compared to the other samples (24%). Nevertheless, increasing the PMeOx/HA unit ratio led to a noticeable increase of the DS except for the highest HA molar masses (i.e. 990 000 g.mol⁻¹). This result suggests that the maximum achievable DS may not exceed the range of 11-13 %, which can be explained by the high steric hindrance induced by both the bulky polyoxazoline functionalities on neighbouring carboxylate moieties and by HA itself. **The starting HA molar mass did not significantly impact the outcome of the reaction. On the other hand,** increasing the DP_{PMeOx} of the grafted PMeOx chain from 29 to 47 led to lower grafting efficiencies when applying similar reaction conditions

as can be seen in Table 2 (e.g. from 16 % for sample HA_{140k}-g-PMeOx₂₉-0.08 to 8 % for sample HA_{140k}-g-PMeOx₄₇-0.04, which were both obtained using a PMeOx (chain) to HA (unit) ratio of 0.5). Finally, the mass yield of the synthesis ranged from 40 to 72% which is satisfying considering the complete purification.

Table 2. Experimental conditions of PMeOx grafting to HA and experimental grafting rates.

Samples	$n_{\text{PMeOx (chain)}}/n_{\text{HA(unit)}}^a$	DP _{PMeOx}	DS ^b	Grafting efficiency (%) ^c	Mass yield (%)
HA _{38k} -g-PMeOx ₄₇ -0.05	0.5	47	0.05	10	50
HA _{38k} -g-PMeOx ₂₉ -0.09	1.0	29	0.09	9	62
HA _{140k} -g-PMeOx ₄₇ -0.04	0.5	47	0.04	8	62
HA _{140k} -g-PMeOx ₂₉ -0.08	0.5	29	0.08	16	n.d.
HA _{140k} -g-PMeOx ₂₉ -0.13	1.0	29	0.13	13	40
HA _{990k} -g-PMeOx ₄₇ -0.05	0.5	47	0.05	10	82
HA _{990k} -g-PMeOx ₂₉ -0.12	0.5	29	0.12	24	72
HA _{990k} -g-PMeOx ₂₉ -0.11	1.0	29	0.11	11	58

^a molar ratio

^b determined by ¹H NMR spectroscopy according to Eq. 1

^c defined as $\frac{DS}{n_{\text{PMeOx(chain)}}/n_{\text{HA(unit)}}}$

n.d.: not determined

The different HA-g-PMeOx were analysed with on-line coupled SEC/MALS/VD/DRI (Fig. 4). The resulting chromatograms display one distribution between 13 and 17 mL of elution corresponding to the grafted HA-g-PMeOx copolymer. SEC/MALS/VD/DRI was found out to be an effective method to check the HA derivative purity. Indeed, free PMeOx, with a much shorter chain than HA, would appear as a secondary distribution at around 20 mL (Fig. S2). For instance, HA-g-PMeOx was still displaying two distributions after the dialysis and subsequent freeze-drying steps and before the washing steps in ethanol (Fig. S3), indicating the dialysis step did not fully remove free PMeOx, despite the membranes cut off (6-8 kDa or 12-14 kDa) being higher than the PMeOx molecular weight (either 2.5 kDa or 4 kDa). This prompted us to additionally wash the material three times in ethanol, after which free PMeOx content was reduced to trace level (i.e < 2 % of the total sample weight) (Fig. 4).

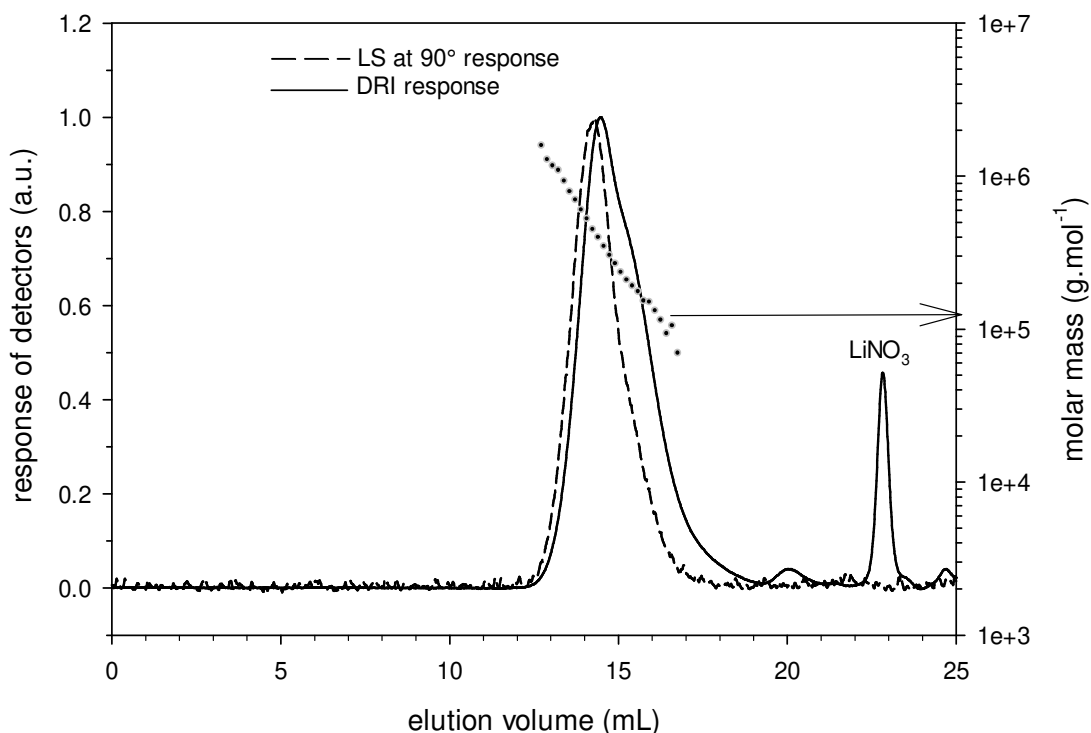


Fig. 4. Elution profiles and molar masses distribution of HA_{140k}-g-PMeOx₂₉-0.13 in 0.1 mol.L⁻¹ LiNO₃ aqueous solution.

In order to further confirm the successful covalent grafting of PMeOx to HA and the absence of remaining free PMeOx after the washing steps with ethanol, diffusion-ordered NMR spectroscopy (DOSY) can be used for differentiating the species in solution according to their size *via* diffusion measurements. DOSY NMR measurements were then undertaken on PMeOx, HA, HA-g-PMeOx polymers (Fig. 5 and 6) and HA / PMeOx physical mixture (Fig. S4 and 6). Unfortunately, in the range of 3-4 ppm, the proton signals of the two polymers were too close to give two different diffusion coefficient values. We decided to focus our attention on the isolated signals assigned to the HA anomeric protons at 4.38-4.32 ppm and PMeOx methyl end group at 2.92-2.88 ppm. For instance, the 2D ¹H DOSY spectra of HA_{140k}, PMeOx₂₉ polymers and the physical mixture of these two polymers HA_{140k}/PMeOx₂₉, show that, as expected, HA_{140k} diffuses more slowly than PMeOx₂₉ and both polymers diffuse much more slowly than H₂O (Fig. 6 a-d-e).

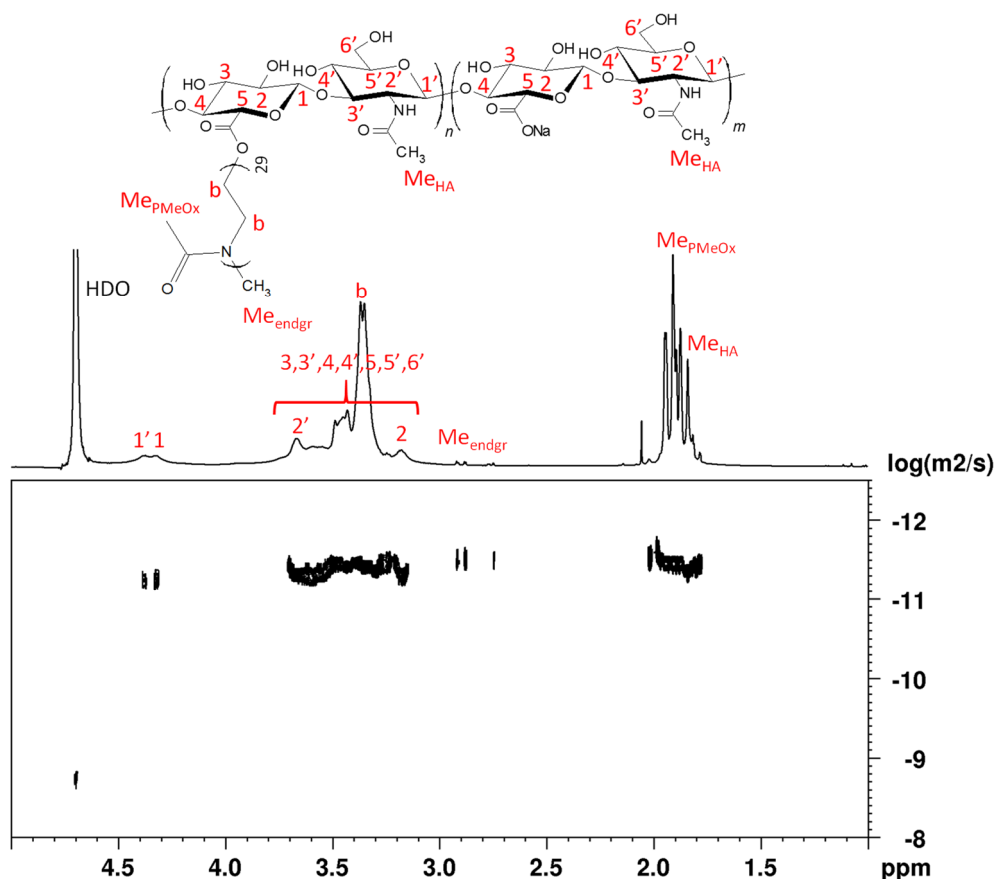


Fig. 5. 2D ^1H DOSY NMR spectrum of $\text{HA}_{140\text{k}}\text{-g-PMeOx}_{29-0.13}$ in D_2O at 290 K.

In the case of the HA-g-PMeOx copolymer, contrary to what was observed on the physical mixture, the 2D ^1H DOSY spectrum shows similar diffusion for the HA anomeric protons and the PMeOx methyl end chains (Fig. 6 a-b-c). This observation is consistent with the behaviour of the two polymers as a single unit and is a further proof of the successful grafting of PMeOx to HA through the formation of a covalent bond and that the sample was well purified.

3.3. Physico-chemical properties of HA-g-PMeOx

The physico-chemical properties of all the samples, obtained with SEC/MALS/VD/DRI analysis, are shown in Table 3.

For $\text{HA}_{38\text{k}}$ and $\text{HA}_{140\text{k}}$, the molar masses increased with the DS, which was expected as the average repetitive unit molar mass became higher when grafted with PMeOx chain [26]. Furthermore, the DP_n (corresponding to the “degree of polymerisation” of HA-g-PMeOx) could be calculated according to both grafted PMeOx M_{nPMeOx} and DS (see Supplementary information for the calculation of DP_n). As a matter of fact, it appeared that for $\text{HA}_{38\text{k}}$ series the

DP_n was quite unchanged after the functionalization except a very slight decrease and consequently a slight degradation for HA_{38k}-g-PMeOx₄₇-0.05, where the DP_n went from 95 to 77. For HA_{140k} series, a noticeable decrease of the DP_n was observed from 350 for the precursor to 270, 300 and 330 for HA_{140k}-g-PMeOx₄₇-0.04, HA_{140k}-g-PMeOx₂₉-0.08 and HA_{140k}-g-PMeOx₂₉-0.13 respectively. This tendency was fully confirmed and amplified with the highest HA molar masses series (i.e. HA_{990k}-g-PMeOx). These results can be correlated to an important HA chain degradation. The longer the starting HA chain, the higher the decrease of molar masses. This appears in agreement with both the increase of flexibility of the chain [28] and the amplified effect of one chain breaking when increasing the chain length (i.e. molar masses). Such degradation could be the consequence of the reaction temperature (60 °C), HA being sensitive to high temperatures [20].

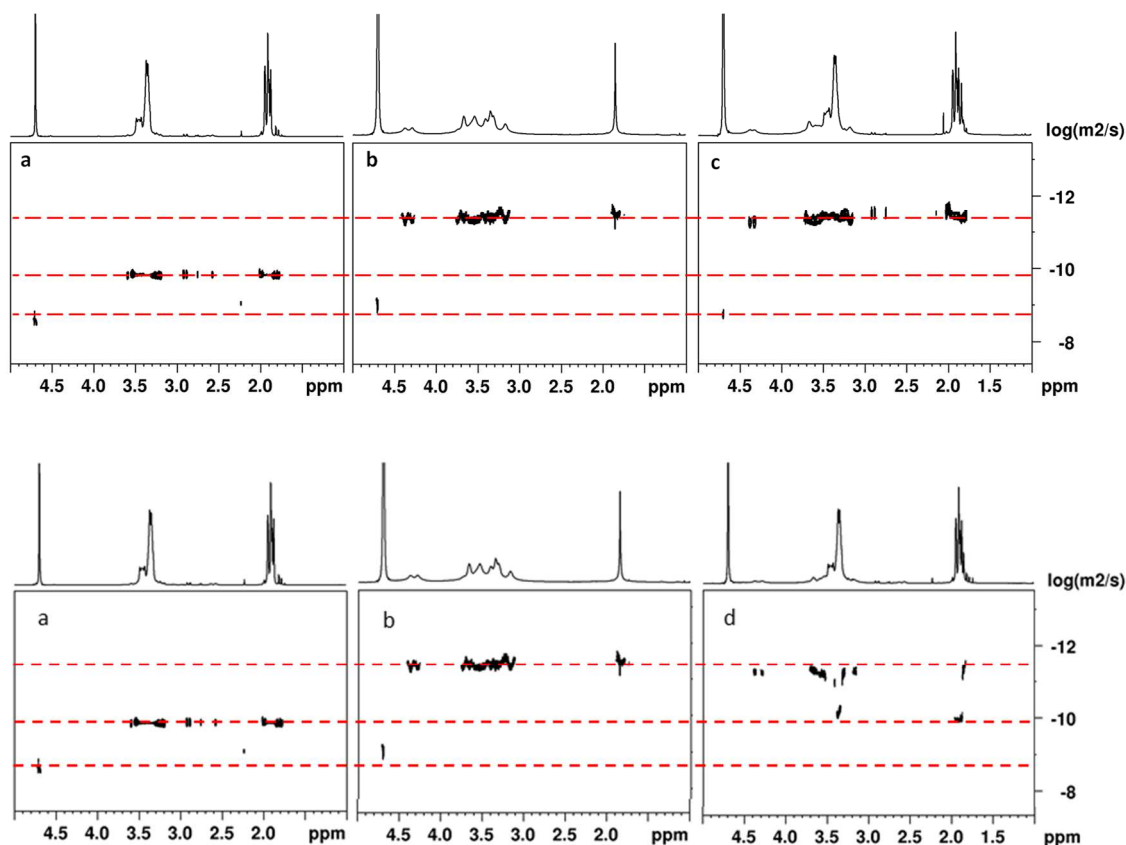


Fig. 6. 2D ¹H DOSY NMR spectra (600 MHz, D₂O, 290 K) of PMeOx₂₉ (a); HA_{140k} (b); HA-g-PMeOx (c); physical mixture of HA_{140k} and PMeOx₂₉ (d).

It must also be noticed that HA₉₉₀ under its TBA-HA form was more difficult to solubilize in DMSO than the HA of lower molar masses, and has been consequently placed at 60 °C for one more day this treatment helping to homogenize the solution. This may also partly explain the highest chain degradation observed for the HA₉₉₀-g-PMeOx samples.

Table 3. Macromolecular characteristics of the samples by SEC/MALS/VD/DRI (25 °C, LiNO₃ 0.1 mol.L⁻¹).

Samples	M _n (g.mol ⁻¹)	M _w (g.mol ⁻¹)	DP _n	Đ	[η] _w (mL.g ⁻¹)	Rh _w (nm)	Rg _w (nm)	a (MHS)
HA _{38k}	38 000	64 000	95	1.68	145	11	^a	0.76
HA _{38k} -g-PMeOx ₄₇ -0.05	46 000	78 000	77	1.68	130	11	^a	0.78
HA _{38k} -g-PMeOx ₂₉ -0.09	60 000	103 000	96	1.70	130	12	^a	0.72
HA _{140k}	140 000	210 000	350	1.50	410	23	44	0.71 ^b
HA _{140k} -g-PMeOx ₄₇ -0.04	150 000	210 000	270	1.41	350	22	38	0.66 ^b
HA _{140k} -g-PMeOx ₂₉ -0.08	180 000	260 000	300	1.45	340	23	38	0.67 ^b
HA _{140k} -g-PMeOx ₂₉ -0.13	240 000	350 000	330	1.45	350	26	45	0.69 ^b
HA _{990k}	990 000	1 300 000	2 475	1.36	1 600	68	147	- ^c
HA _{990k} -g-PMeOx ₄₇ -0.05	640 000	940 000	1065	1.47	890	49	94	- ^c
HA _{990k} -g-PMeOx ₂₉ -0.12	470 000	650 000	675	1.37	570	38	66	- ^c
HA _{990k} -g-PMeOx ₂₉ -0.11	650 000	910 000	967	1.40	750	46	80	- ^c

MHS coefficient (a) has been determined by integrating the most linear part of bilogarithmic representation of intrinsic viscosity vs molar mass

SEC/MALS/VD/DRI data accuracy: M_n ± 9%; M_w ± 6%; Đ ± 10%; [η]_w ± 0.5%; Rh_w ± 2%; Rg_w ± 6%

^a out of the range of the MALS detector (isotropic diffusion for lower masses fractions)

^b See Fig. S5 for MHS plots

^c undetermined values due to challenging analysis

Other physico-chemical parameters could also be determined by SEC/MALS/VD/DRI (Table 3): gyration radii (Rg_w), intrinsic viscosities ([η]_w), hydrodynamic radii (Rh_w) and the Mark Houwink Sakurada (MHS) parameter 'a' (Mark-Houwink-Sakurada plots are given in Fig. S5). One can observe the decrease of size parameters (Rg_w, [η]_w and Rh_w) correlated with the above discussed degradation. For instance, Rg_w, [η]_w and Rh_w values of HA_{990k}-g-PMeOx₂₉-0.11 (respectively 80 nm, 750 mL.g⁻¹ and 46 nm) were all lower than those of starting HA_{990k}

(respectively 147 nm, 1600 mL.g⁻¹ and 68 nm) which correlated well with the drop of the DP_n of the polysaccharide chain (from 2475 to 967 after modification). Concerning the MHS parameter, which gives conformational information, the results seem to indicate that the HA-*g*-PMeOx derivatives present quite the same conformation than the corresponding HA precursors. Meanwhile 'a' values were always around 0.7 which means all polymers present a classical random coil conformation in good solvent [28]. Logically, PMeOx moieties are clearly hydrophilic and do not affect the water-soluble character of HA derivatives.

3.4. Application of the reaction to devise HA-based physical hydrogels

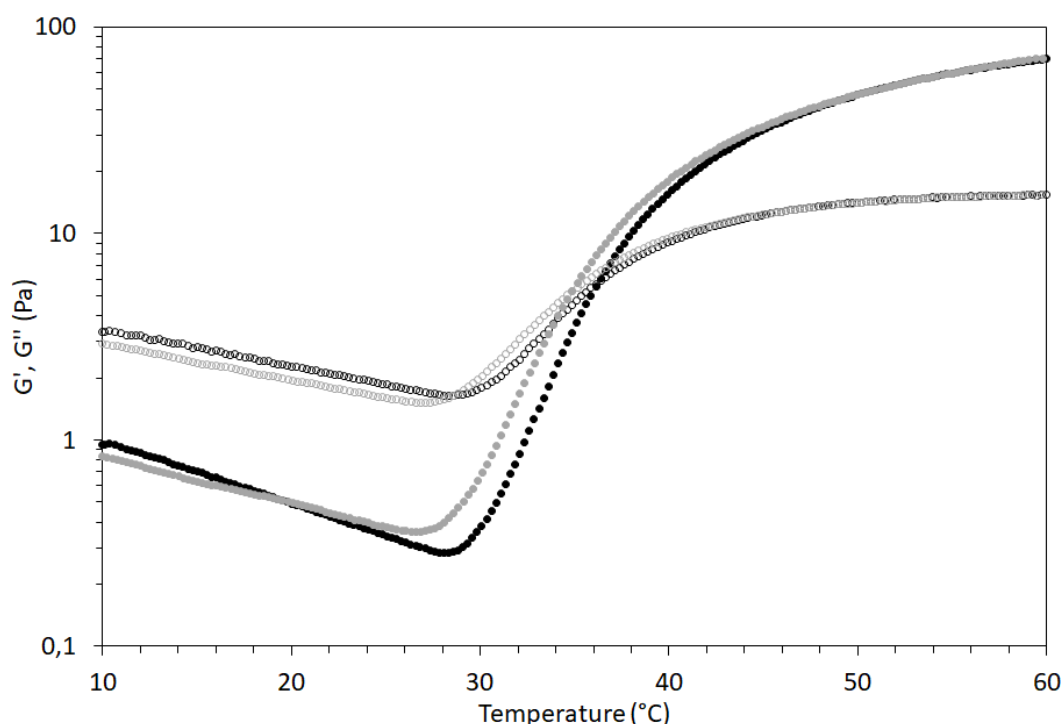


Fig. 7. Rheological profile of HA_{990k}-*g*-(PiPrOx₂₀-*co*-PnBuOx₇)-0.10 (*G'*: full circles; *G''*: empty circles; heating ramp: black circles; cooling ramp: grey circles) at 1.5 wt% in PBS during a temperature ramp (rate: 0.5 °C.min⁻¹; shear stress: 0.1 Pa; frequency: 1Hz).

The poly(2-alkyl-2-oxazoline) polymerisation reaction was then performed on a mixture of *i*PrOx and *n*BuOx, the resulting copolymer, PiPrOx₂₀-*co*-PnBuOx₇ (DP: 27), being thermo-responsive [7] [16], with a cloud point at 26 °C for 10 g.L⁻¹ solutions) or at 32 °C for 2 g.L⁻¹ solutions according to our measurements. It was subsequently terminated with HA_{990k} to result in HA_{990k}-*g*-(PiPrOx₂₀-*co*-PnBuOx₇)-0.10 (see Fig. S6 for ¹H-NMR spectrum), which was then dissolved in PBS as a 1.5 wt% solution to perform rheological measurements on it. Briefly, a

0.5 °C.min⁻¹ heating ramp was applied on the solution from 10 °C to 60 °C with a set stress of 0.1 Pa (linearity domain was checked beforehand) and a set frequency of 1 Hz, followed by a corresponding cooling ramp. The evolution of the storage modulus G' and of the loss modulus G'' with temperature were then plotted, the results being displayed on Fig. 7. A reversible thermal transition was clearly seen, with G' being below G'' at the lower temperatures, indicative of a predominantly viscous behaviour, before crossing it at 36.2 °C (34.9 °C during the cooling ramp) and eventually becoming higher at the higher temperatures, indicative of a predominantly elastic behaviour typical of hydrogels.

This result is quite promising for us and we will investigate the influence of the grafted copolymer composition and length on the functionalized HA hydrogel transition in an upcoming publication.

4. Conclusion

PMeOx was successfully grafted on HA through a direct termination route between the oxazolinium terminal group of PMeOx chain and carboxylate moieties of HA without using any activating agent. The successful grafting as well as the purity of the resulting HA-*g*-PMeOx have been confirmed with SEC/MALS/VD/DRI and DOSY NMR experiments. The one-pot functionalization has been conducted with various lengths (DP_{PMeOx}) of PMeOx chain and/or target DS on various HA molar masses. The DS seems to cap up to about 13 % probably due to the steric hindrance of both PMeOx and HA. Nevertheless, it should be noted such a DS is already accounting for a PMeOx weight fraction in HA-*g*-PMeOx of 45 %. Characterisation of the grafted HA derivatives indicated a HA chain degradation under high temperature (i.e. 60 °C) that was more pronounced when HA molar mass increases. From a conformational aspect based on the MHS exponent analysis, HA derivatives (HA-*g*-PMeOx) and precursors (Na-HA) seem to present the same random coil conformation in water (i.e. good solvent).

The reaction could then be extended to another poly(2-alkyl-2-oxazoline), namely $PiPrOx_{20}-co-PnBuOx_7$, which was terminated with HA_{990k}, the resulting copolymer showing a hydrogel transition around 36 °C as a 1.5 wt% solution in PBS. Future work will further focus on the HA grafting with thermo-responsive poly(2-alkyl-2-oxazoline). Both polymers are highly biocompatible, HA being a component of the extracellular matrix and poly(2-alkyl-2-oxazoline) being increasingly used for biomedical applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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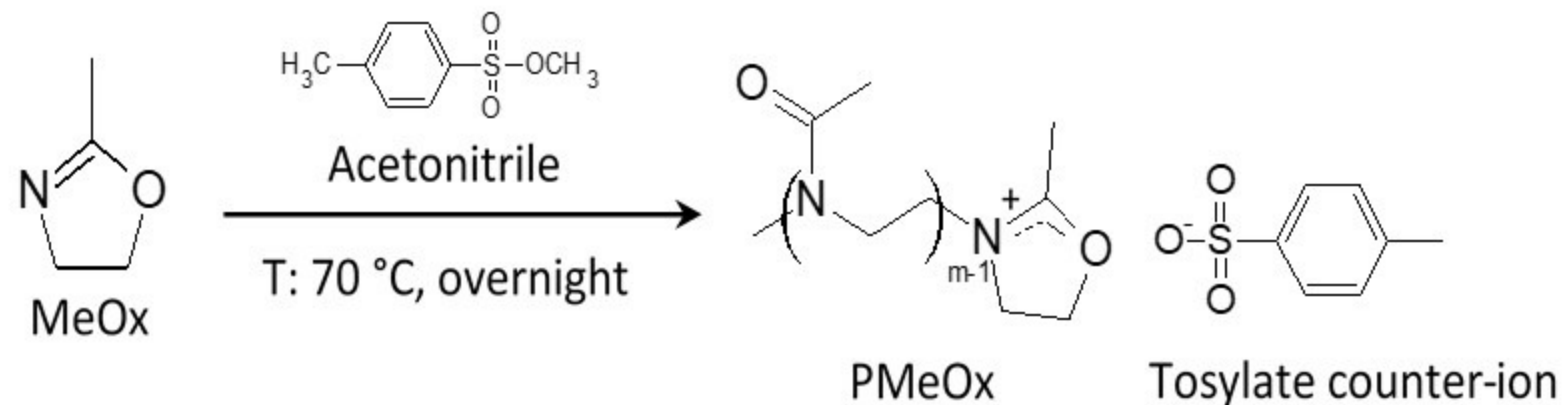
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Polymerization of MeOx

Termination step with TBA-HA



One-pot functionalization of HA



HA



HA-g-PMeOx

Random coil
conformation
with no free
PMeOx